INTRODUCTION

1.1 Facts about Plant Tissue Culture

The success of plant biotechnology relies on the techniques of plant tissue culture. For proper utilization of plant system, knowledge of basic biology of plant is an important need. Plant tissue culture provides a basic knowledge of physical and chemical requirements of cell, tissue, organ culture, growth and development. Development of cell, tissue, organ culture and regeneration of plantlets under *in vitro* condition has opened up a new horizon in the areas of plant biotechnology.

The Science of plant Tissue culture takes its root from the path breaking research in Botany like discovery of cell and subsequent propounding of cell theory. Schleiden and Schwann in 1839 proposed that cell is the basic unit of life and it has a ability to regenerate into a whole plant if placed in a condition favorable to regeneration. This concept is known as totipotency which is the basis for plant tissue culture and the term totipotency was coined by Steward in 1968. Based on this Gottlieb Haberlandt developed the concept of *in vitro* cell culture in 1902.

Plant tissue culture is the culture and maintenance of plant cells in sterile, nutritionally and environmentally supportive conditions. It is a method of culturing cells, tissues and organs on synthetic media under aseptic environment and controlled condition of temperature,light and humidity. The development of plant tissue culture as a fundamental science was closely associated with the discovery and characterization of plant hormones which has facilitated our understandings of plant growth and development. *In vitro* multiplication of plants by culturing tissues to produce copies of a parent plant which are genetically identical is referred as micropropagation or clonal propagation. This technique helps to generate genetically similar copies of parent plant thus it permits continuation of the parental characters of the cultivars among the plants generated from micropropagation. *In vitro* propagation forms the basis of a large number of practical applications in agriculture, horticulture, industrial chemistry and is a prerequisite for plant genetic engineering.

Plant tissue culture is a technique of *in vitro* cultivation of plant cells and organs , which divide and regenerate into callus or plant organs. Technique of tissue culture relies on the following facts:

- Explant
- Aseptic condition
- Nutrient media

A small piece of tissue cut from any part of the plant is called explant. Choice of explants varies with species. Meristematic parts are actively dividing for that they are more responsive and give better success as explants.

During culture aseptic condition is required to avoid contamination from microorganisms. All the materials like glassware, instruments, media, explants to be used during culture work must be sterilized using several techniques available for sterilization purpose.

The selection of tissue culture medium largely depends upon the species to be cultured. For example some tissues show better response in solid medium some show better response in liquid medium. The type and composition of culture media very strongly effect the growth and morphogenesis of plant tissues. The most suitable and commonly used medium is Murashige and Skoog (MS) medium for plant regeneration from tissues and callus. MS media is a high salt medium due to its content of potassium and nitrogen salts. In protoplast culture B5 medium is effective. It has lesser amounts of nitrate and ammonium salts than MS medium. For anther culture Nitsch's medium is most effective.

1.2 Components of Tissue Culture Medium:

1. Inorganic Nutrients: *In vitro* growth of plants requires combination of macro and micronutrients. Macronutrients are categorised as those elements which are required in concentration greater than 0.5mM/l. These are nitrogen, potassium, phosphorous, calcium, magnesium and sulphur in form of salts in media. Micronutrients are those elements which required at a concentration less than 0.05mM/l. These include iron, manganese, zinc, boron, copper and

molybdenum. Iron is provided as iron EDTA complex to make it available at wide range of pH.

2. Carbon Source: Sugar play an very important role as energy source in nutrient media because most plant culture unable to photosynthesize owing to inadequately developed cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels etc. The most preferred carbon source is sucrose at concentration of 20-60g/l.

3. Organic Supplements:

a. Vitamins: Vitamins are the organic substances required for metabolic processes as cofactor or parts of enzymes. For optimum growth, medium should be supplemented with vitamins.

b. Amino acids: Incorporation of amino acids to media is important for stimulating cell growth and protoplast cultures and also inducing and maintaining somatic embryogenesis. This reduced organic nitrogen is more readily taken up by plants than the organic nitrogen. L-glutamine, L-asparagine, L-cystein, L-glycine are commonly used amino acids in tissue culture medium.

c. Complex organics: A group of undefined supplements such as casein hydrolysate, coconut milk, yeast extract, orange juice, tomato juice etc are known as complex organics. These compounds are often used when other combination of known defined components are fail to produce the desired growth.

d. Activated Charcoal: Activated charcoal acts both in promotion and inhibition of culture growth depending upon the plant species being cultured. It reduce toxicity by absorbing brown-black pigments and oxidized phenolics produced during culture. It also absorbs other organic compounds like PGRs, vitamins etc which may cause growth inhibition. Activated charcoal helps root formation and growth by darkening the medium.

e. PGRs: Plant growth regulators stimulate cell division and hence regulate growth and differentiation of shoot and roots on explants and embryos in *in*

vitro cultures. The four major plant growth regulators used in tissue culture are auxin, gibberelline, cytokinin and abscissic acid.

I. Auxins : Auxins induce cell division cell elongation, apical dominance, adventitious root formation and somatic embryogenesis. At low concentration auxins induce root initiation and at high it helps in callus formation. Most commonly used synthetic auxins in *in vitro* culture are 1-napthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), indole -3acetic acid (IAA),indole butyric acid (IBA)etc.

II. Cytokinins : Cytokinins induce cell division and initiation and growth of shoots *in vitro*. Zeatin, 6-benzylaminopurine (BAP), kinetin, 2-ip are the mostly used cytokinins. They modify apical dominance by promoting axillary shoot formation. Cytokinins inhibit root formation and induce adventitious shoot formation when used in high concentration.

III. Gibberllins : Gibberellic acid (GA3) is used for internode elongation and meristem growth.

IV. Abscissic acid: Abscissic acid is used only for culturing woody species and somatic embryogenesis.

5. Solidifying agents: Solidifying agents are used for preparing semisolid tissue culture media. Agar can bind water and it is added to the medium in concentration ranging from 0.5% to 1% (w/v). Agar is preferred over all gelling agents because it is inert, neither does it react with media constituents nor digested by plant enzymes.

Agarose is also used as solidifying agent. Gelrite is a gelling agent which form clear gel and it help to detect contamination easily.

6. pH : Optimum pH for culture media is 5.8 and it affects absorption of ions, solidification of gelling agent. pH value lower than 4.5 and higher than 7.0 greatly inhibit growth and development *in vitro*. After autoclaving the pH of culture media drops by 0.3-0.5 units.

Tissue culture is basically defined as *in vitro* growth and development of plantlets from any part of the plants in suitable nutritive culture medium.In

scientific terminology it is also known as micropropagation. Plant tissue culture is a technique of plant cells, tissues and organs culture in nutrient medium which is prepared artificially under aseptic condition. It helps to improve the knowledge of fundamental Botany such as agriculture, horticulture, plant breeding, forestry, phytopathology and industrial production of plant metabolites, hybridization of somatic cells.Nutrient medium composed of inorganic salts, carbon source, vitamins, growth regulators and organic supplements, is suitable for plant tissue culture.

The ability of a plant cells to regenerate a whole plant is called totipotency and plant tissue culture believe on the fact. Aseptic culture of cells, tissues, organs and their components under controlled physical and chemical conditions *in vitro* is an important tool for basic and applied studies as well as commercial application. *In vitro* propagation finds its path to the ideas of the German scientist, Haberlandt at the beginning of 20^{th} century.A number of necessary information has been added recently to our knowledge of the nutrition of cells, tissues and organs grown *in vitro*. Several improvements have been made in the composition of the media in the last 30 years.

Plant tissue culture is a technique used to propagate plants under sterile conditions to produce clones of a plant. Different techniques used in culturing plant tissue may provide certain advantages which are better than traditional methods of propagation.

1.3 Advantages of Plant Tissue Culture

- The production of exact copies of plants that have some good qualities.
- To quickly generate mature plants.
- The production of plants in sterile containers that allows reducing the chances of transmitting diseases, pests, pathogens.
- The production of plants from seeds which otherwise have very low chances of germinating and growing i.e. orchids and nepenthes.
- To clean particular plant of viral and other infections and to quickly multiply these plants has ‘cleaned stock’ for horticulture and agriculture.

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate vs. ammonium salts or amino acids) have significant influences on the tissue morphology that grow from initial explants. For example, an excess of auxin will often enhance root proliferation while an excess of cytokinin may enhance shoot proliferation.

The tissue obtained from the plants to culture is called an explant. In many species, segment of various organs used as explant; vary in their rates of growth as well as regeneration while some don't grow at all. The difference in regeneration ability of the various organs have various reasons.The reasons behind the differences in regenetation potentiality of various explant include effect in the cell cycle, the ability to transport endogenous growth regulators, and also influence the metabolic capabilities of the cells. The most commonly used tissue explants are the meristematic portion of the plants like the stem tip, axillary bud tip. Meristematic tissues divide rapidly either by concentrating or producing required growth regulating substances including auxins and cytokinins.

Culture of plant tissue is used widely in plant study and it also has a number of commercial applications.Tissues culture consists of growing plants cells on an agar medium as callus known as callus culture and small cell masses in a liquid medium known as suspension culture. Tissue culture is used for vegetative multiplication of many species which are threatened or less ability to propagate naturallty and in some cases to regenerate of disease free plants. It has its application in production of somatic hybrids, genetic transformation, organelle and cytoplasm transfer.

Propagation through *in vitro* culture provides a solution for mass propagation of plants in many rare,threatened endangered or economically important plants in particular. In developing countries medicinal plants are important to the health of many people.

Biodiversity is the term which implies the total sum of variety of lives in a region or indeed across the whole earth. The losses of biodiversity influence directly and indirectly economic consequences, as plants are essential sources of food and medicines.

The over exploitation of plant resources, with increasing habitat destruction and /or fragmentation, pollution and introduction of exotic species are the important reasons for many plant species extinction.Today it is widely accepted that the plant extinction rate has reached one species per day due to many reasons including human activities, the rate is 1000-10000 times faster than would naturally occur otherwise (Hilton-Taylor,2000) and if this process is continue according to the trend it may be the cause of disappearance of between 60000-100000 plant species during the next 50 years (Akeroyd 2000, Bramwell,2002). The latest investigations on the scope of the World's threatened flora suggest that as many as half of the World's plant species may be qualified as threatened by extinction under the IUCN criteria (Pitman and Jørgensen, 2002). The latest survey by the International Union for the Conservation of Nature (IUCN) of more than 12000 species has revealed that nearly 70% of the species are threatened and of that about 19% are critically endangered(CR). More than 50% of plant species are endemic to the 34 Global Biodiversity hotspots and there are 1500 endemic species in each hotspots (Barnicoat,2011).Species which are either extinct in the wild (EW) or critically endangered (CR) are high priority to rescue and conservation using *in vitro* measures (Sarasan *et al*.,2006).

The World Conservation Monitoring Centre listed 82 Pteridophytes species are threatened, or in some cases extinct in India. Jain and Sastry (1980) reported that 17 rare and endangered species of pteridophytes from India. Dixit (1984) reported 25 rare and interesting pteridophytes. Bir (1987a) listed that 104 rare and endangered species of pteridophytes from various regions of India.

As a result of rapid agricultural and urban development, deforestation and indiscriminate collection many important plant species which have medicinal or other economic importance are disappearing at an alarming rate . There is a need to conserve plants with medicinal value. Plant tissue culture technology may have to conserve rare and endangered medicinal plants. Many important Chinese medicinal herbs are successfully propagated by *in vitro* propagation technique. Due to ever growing demand the availability of wild medicinal

plants to the pharmaceutical companies is not enough to manufacture herbal medicines and this crisis can be handled with the help of tissue culture.

In recent times, attention on plant research has increased all over the world and a large number of evidence has collected to reveal the immense potentiality of medicinal plants used in various traditional system to cure diseases. More than 13000 plants have been studied during last five years periods. Medicinal herbs are moving from fringe to main stream use with greater number of people seeking benefits free from side effects. Recently eco-friendly and bio-friendly plant based products are in demand for prevention and cure of various kinds of human diseases including microbial infections. The plant kingdom represents an extra ordinary reservoir of novel molecules. The potentialility of higher plants, as a source for new drug to cure many diseases is still largely unexplored.

The uses of plants, plant extract provide the foundation to modern therapeutic sciences and thus enable the man to establish the empirical system of medicine. In view of the commercial importance given to the secondary metabolites in recent time's efficient production of bioactive compounds by tissue culture technology has gained popularity. The continuous and non organized collection has resulted in many plants becoming rare and some even become extinct. To overcome this limitation, biotechnologist suggested the use of *in vitro* culture to save the extinct species.

Barak valley contains many medicinal rare and endangered plants. These plants are depleting from natural habitat due to several reasons including human activities. It is the need of the hour that all the valuable plants must be conserved. Plant tissue culture technology is the most suitable technique for conservation aspects.

North Eastern India has been very well known for its rich biological diversity and Barak Valley of Assam is very much well known for its plant diversity, specially medicinal plant diversity. A variety of biochemical products are synthesized and preserved by green plants and many of them are used as raw material for various scientific investigations as well as chemical utilization. Medicinal plants are generally known as "Chemical Goldmines" as a large

number of natural chemicals are present in plants. All the chemicals cannot be synthesized in laboratories. Plant's secondary metabolites are commercially important and used in a number of pharmaceutical compounds. Since the beginning of civilization human beings are dependent on plant for their food and health care needs. There are 250000 higher plant species on earth and of them 80000 plants contain medicinal property. About 5000 species are used as medicine in various traditional system. The Red Data Book of India has a list of 427 number of endangered species of plant of which 28 species are treated as extinct, 124 species are threatened, 81vulnerable, 100 rare and 34 insufficiently known species (Thomas, 1997).

One of the richest traditions of ethnobotanical medicine is found in India in the world with more than 700 species of plants used in different indigenous systems of medicine and industries. Over 95% of plants with medicinal property are collected from wild sources by herbal and pharmaceutical industries. The alarming rate of loss of biodiversity due to other known factors with the indiscriminate collection of wild plants with medicinal property and there lies a real threat of extinction of many of our medicinal plant species. In the phase of serious threat to biodiversity loss, it is extremely necessary to take immediate steps to conserve the genetic resources of medicinal plants.

According to the World Resource Institute, India place among 28 countries that are facing severe effects of increasing ecological imbalance. The IUCN Report reveals that in India 7.7% of the plants are under threat. In conservational aspect plant tissue culture is an effective tool to conserve the plant genes and guarantee the survival of the endemic, endangered and over exploited genotypes. It uses small units (cell, tissue) without losing the mother plant.

1.4 Physiographical features of Barak Valley

Barak valley is named after its important river 'Barak' which is originated from the Naga and Manipur hills and it is the southernmost part of Assam. This part of Assam (Barak valley) is situated within 24°80□-20°04□N latitude and 93°15□-90°44□E longitude having a total area of about 6951 sq. km (Cachar = 3786 sq km, Karimganj= 1839 sq. km, and Hailakandi = 1326

sq. km).The altitude of the Barak Valley rises upto 1400m above msl (Das, 2011). The Valley is surrounded by North Cachar hills district of Assam and Jaintia hills of Meghalaya in North side, by Mizoram in the South side, by Manipur state in the East side and by Tripura state and partly by Sylhet district of Bangladesh in west side (Chakraborty *et al.,* 2010).

The climatic condition of Barak valley is sub-tropical, warm and humid. The temperature ranges between 9.2°C to 36.9°C. Distribution of rainfall of the Valley maintain a definite pattern – a high rain fall zone (above 4000 mm) in the north western part with high hill areas, the medium rainfall zone (3000-4000mm) covering the largest area in the central region and low rain fall zone (below 3000 mm) in the entire southern part of the valley covering Manipur, Tripura and Mizoram. The growth of diverse vegetation is favored by total annual rain fall which is adequate in this zone. The Barak valley can be differentiated into High hill region, Dissected foothill region, Low hill region, Undulating plains, Detraited Valleys, Broad meander plains, flood plains and low lying areas known as beels and hoars (Dutta Choudhury *et al*., 2009). The major soil types of the Valley are Old riverine alluvium soil, old mountain alluvium, non laterised soil, laterised red soil and peat soil. The pH of the soil ranges from 4.4 - 6.0 which indicate acidic soil.

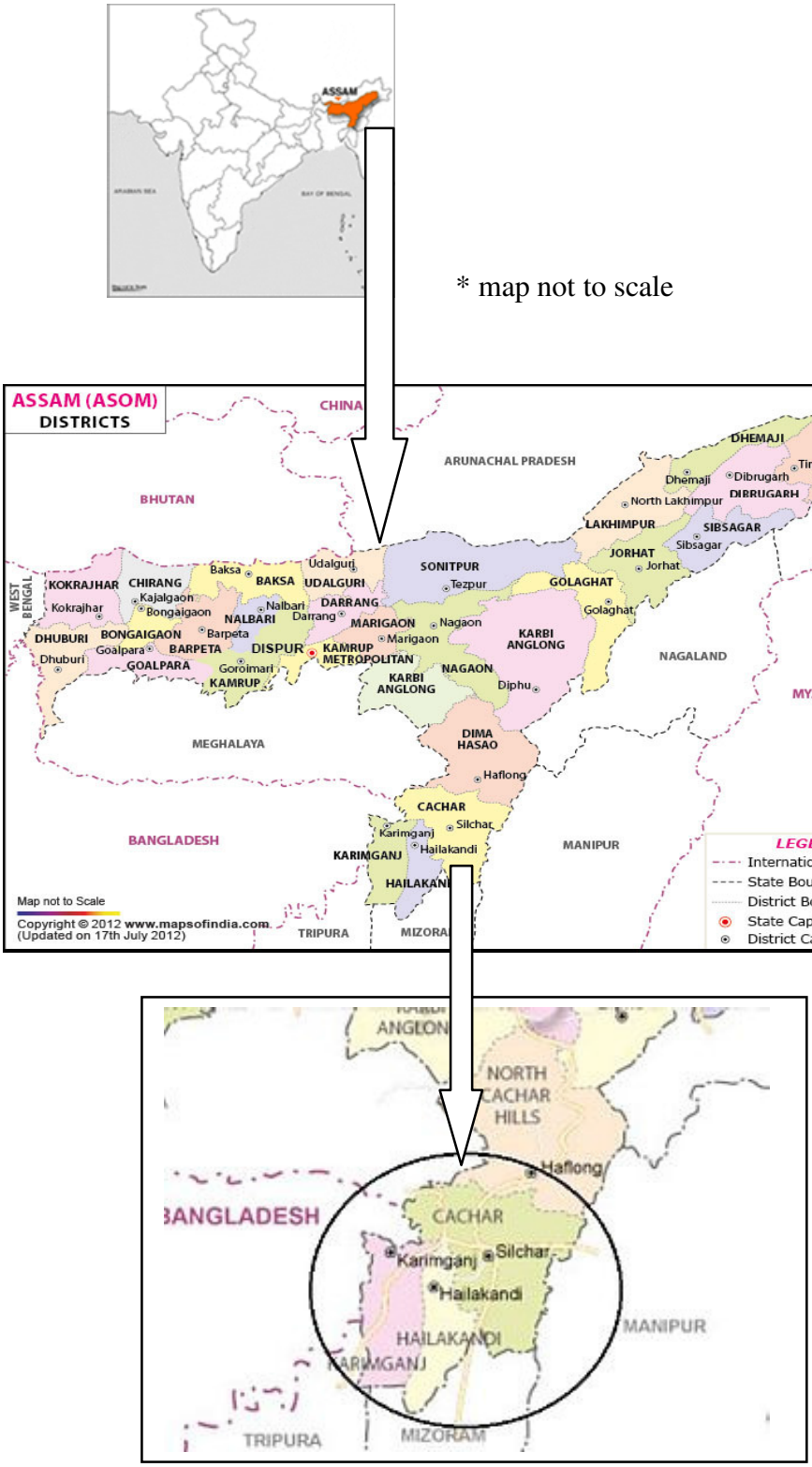

Figure1. Maps showing study sites